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Microorganisms - Tools for Bioremediation of Uranium Contaminated Environments

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Abstract

Uranium contaminated sites harbor viable and metabolically active microorganisms, capable of interacting with uranium and impacting its distribution. This study wants to contribute to a better understanding of the microbe-uranium interactions in the Urgeiriça mine (Portugal) by enumerating, identifying and characterizing U resistant strains. *Rhodanobacter* genus included a major group of U (VI) resistant isolates up to 2 mM U (VI). The analysis suggests that different species belonging to this genus have different resistance profiles. The recovery of these bacteria as a major group of isolates indicate that these microorganisms might have an important ecological role in these environments.

Keywords: Rhodanobacter, uranium, mine, metal resistance

1. Introduction

Uranium is the most abundant of the naturally-occurring actinides with concentrations of 1 to 4 ppm in crustal rocks and sediments[1]. Uranium in the environment occurs primarily as 3 of its 17 known isotopes, ^{238}U (99.27%), ^{235}U (0.72%), and ^{234}U (0.005%). All are radioactive; however, is the chemical toxicity of this element that is of

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greatest ecological concern[2]. Uranium at contaminated sites has two major oxidation states, U (IV) and U (VI). U (IV) is highly insoluble and usually precipitates as uraninite (UO_2). U (VI) often forms complexes with high solubility and mobility, and therefore is considered to be more toxic in the environmental systems[2,3]. The main uranium mining facilities in Portugal were located at the Urgeiriça mine that presently is closed. Former uranium mines like the Urgeiriça are a source of environmental contamination, since most of them continue to discharge uranium via acid mine drainage water, resulting in soil, subsoil and groundwater contamination. Currently, the Urgeiriça mine is under a process of environmental remediation. The treatment of the contaminated acidic waters consists of the addition of barium chloride to precipitate the metals and radionuclides, which are accumulated in sludge lagoons. These sludges are removed to specific landfills for contaminated materials, leaving unsolved the potential for environmental contamination. Thus, an improvement of the efficiency of the treatment lagoons and their long-term stabilization by effective and low cost techniques will have a positive environmental impact in the Urgeiriça mine surrounding areas. It has been demonstrated that uranium contaminated sites often harbor viable and metabolically active microorganisms capable of interacting with uranium by different mechanisms that dramatically impact the form and distribution of uranium in the environment[4-6]. In this sense, this study wants to contribute to a better understanding of the microbe-uranium interactions in the Urgeiriça mine by enumerating, identifying and characterizing U resistant strains for their potential future use as in situ bioremediation tools.

2. Material and methods

2.1 Site description and sample collection

The Urgeiriça mine is currently deactivated and a program of environmental remediation is under development. At the time of sampling, the two most important focus of possible environmental contamination in the mining area were the underground mine water and water from the rain that percolates the tailing material of Barragem Velha with exsurgences in the valley of a nearby river (Ribeira da Patranha) (Fig. 1). In order to prevent contamination of the river, a water treatment plant system was developed in the area called Barragem Nova. Basically, the waters in the mining area and the acid exsurgences originated from Barragem Velha were collected and diverted to the Barragem Nova treatment plant. In the water treatment system, water was neutralized through the addiction of burn lime. Barium chloride was also used to induce precipitation of radium compounds and to remove heavy metals including uranium. Water was then kept for some time in a settling basin (sludge lagoon) for pour off the precipitated chemical compounds which gradually were accumulated at the bottom; finally, it was discharged in the Ribeira da Patranha stream at the exit of the mining area.

Water and sediments were collected from relevant sources of the natural system, contamination focus and from different sites of the water treatment plant. Namely, sample A6; water and sediments from Ribeira da Patranha stream before reaching the mining area; sample 7A; underground water from the mine; sample A2; untreated water from the mine and exsurgences collected in Poço das Cobras; sample A4 sludge from the settling basin and sample A5; water released in the Ribeira da Patranha stream after depuration in the treatment system[1].

2.2 Chemical and radiological analysis of sampling sites

The pH and the temperature of the samples were measured at the time of collection. The total uranium content of the samples was measured by liquid scintillation counting. The counting of the decay of the U isotopes was performed using an ultralow level spectrometer, Quantulus 1200, from Perkin-Elmer, using an alpha–beta discriminator and controlling the quench effect through the external standard method. The background was evaluated by counting, under the same conditions and time, a blank vial filled with the same scintillation cocktail. The average count for the blank was 0.723 cpm (counts per minute). A spike of a natural U standard, supplied by Ciemat (Spain), was used to calibrate the window of the multichannel analyzer as well as the alpha-beta discriminator. Under these experimental conditions, the measured total count is directly proportional to the activity of the U isotopes U-238 and U-234[7].

2.3. Enumeration and isolation of the heterotrophic and metal-resistant populations

Viable, metal resistant bacteria populations of the samples collected in the Urgeiriça area were enumerated by CFU (colony forming unit) counts. In duplicate, 0.1 ml of diluted samples, in NaCl 0.85%, (up to 10^{-3}) were spread on R2A medium[8] supplemented with 1 mM of chromate (VI), or 3 mM of arsenic (III) or 2 mM of uranium (VI). The samples were incubated at the temperature of 22°C and pH of 7.0 for up to 10 days. To avoid the growth of fungi on the plates, the R2A medium for isolation of strains was supplemented with cycloheximide 0.7 % (w/v). The cultures were observed every two days for enumeration, and different morphological colony types were isolated. Isolates were purified by subculturing and preserved for further studies at - 80°C.

2.4 Tolerance to different metals of the isolated population

Bacteria recovered on the different media were evaluated to their metal resistance. The tolerance to different metals was tested in R2A medium supplemented with chromium (VI) 1 and 2 mM; uranium (VI) 1 and 2 mM; cobalt (II) 1 and 2 mM; copper (II) 1 and 2 mM; arsenic (III) 2 and 4 mM; zinc 5 and 10 mM; and antimony (V) 5 and 10 mM.

2.5 Phylogenetic characterization of a major population of Rhodanobacter strains

DNA was extracted, the 16S rRNA gene was amplified and the PCR products were purified and sequenced as described[9]. The quality of the 16S rRNA gene sequences was manually checked using the Sequence Scanner Software V 1.0 (Life technologies Corporation, Carlsbad, CA, USA). The sequences generated in this study and their closely related reference sequences were aligned using the SINA aligner[10] and a neighbor-joining phylogenetic tree was constructed using the MEGA (5.1) program package[11].

3. Results

3.1 Chemical and radiological analysis of sampling sites

Concerning the chemical and radiological analysis of the sampling sites, only total uranium concentration, pH and temperature values were determined (Fig. 1). The uranium concentration was higher (8.2 μM) in the sampling site A2 when compared with the other Urgeiriça sampling sites (<0.01-3.0 μM). The temperature (19-21°C) and pH (pH=6) were very similar in all the sampling sites with exception of A2 and A4, which had a lower pH (pH=5) and a higher pH (pH=9), respectively.

3.2 Enumeration and isolation of heterotrophic bacteria in Urgeiriça samples

The cultivable fraction of microbial populations of the Urgeiriça samples was enumerated by colony forming units (CFUs) counts on R2A medium at temperature of 22° C. The number of CFUs recovered on R2A medium supplemented with 2 mM of U (VI) or 3 mM of Ars (III) ranged between 1.0×10^2 CFU ml^{-1} and 2.8×10^3 CFU ml^{-1} (Fig. 1). In presence of 1 mM of Cr (VI) there was no colony forming.

A total of 70 isolates were recovered from the Poço das Cobras, the site with higher uranium content. Among these isolates, a major group of 9 strains resistant to U (VI) were identified as *Rhodanobacter* sp. by partial sequencing of the 16S rRNA gene and were selected for further studies.

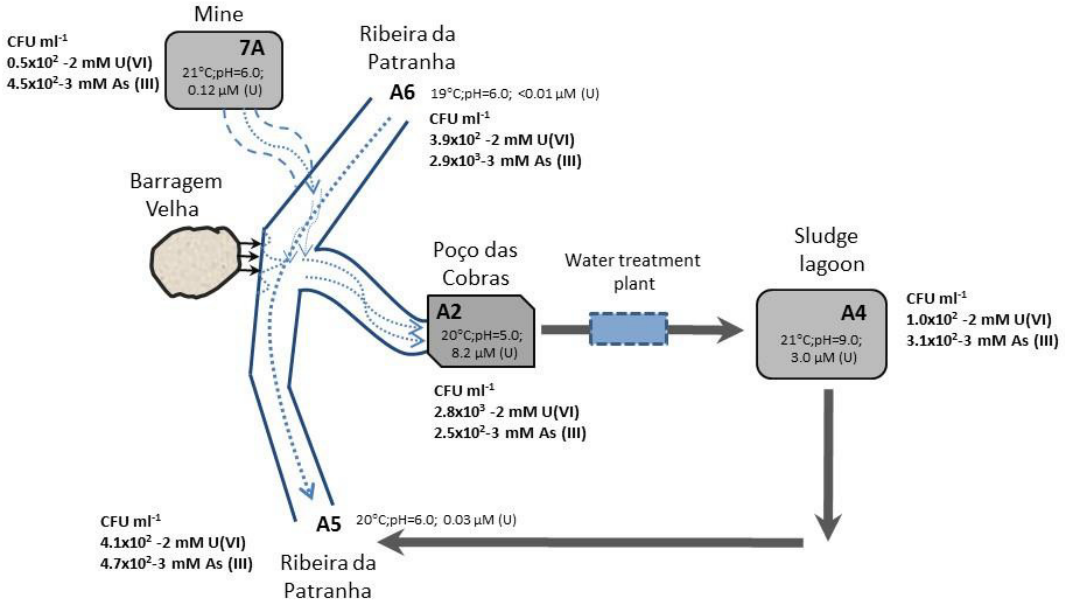

Fig. 1. Schematic represenation of the sampling sites.
Temperature (ºC), pH and U total (µM) is reported for each sampling site. Metal and metalloid-resistant populations were enumerated by colony forming units (CFUs) counts on R2A medium supplemented with 2 mM of U(VI) and 3 mM of As(III), T = 22°C and pH = 7.0.

3.3 Metal resistance phenotype of Rhodanobacter strains

The metal resistant phenotype of *Rhodanobacter* strains is presented in Table 1. All except strain A2-441 were resistant to 2 mM U (VI) and strain A2-71 was the only strain resistant to Cr (VI). None of the strains were able to resist to 2 mM of Co (II) or Cu (II).

Table 1. Metal resistant phenotype of *Rhodanobacter* strains used in this study.

Strains	Cr (VI.) mM		As (III) mM		U (VI) mM		Zn (II) mM		Sb (V) mM		Co (II) mM		Cu (II) mM	
	1	2	2	4	1	2	5	10	5	10	1	2	1	2
A2-56	-	-	-	-	+	+	-	-	+	+	-	-	-	-
A2-60	-	-	-	-	+	+	+	-	+	+	-	-	+	-
A2-61	-	-	+	+	+	+	+	+	+	+	-	-	+	-
A2-69	-	-	-	-	+	+	+	-	+	+	-	-	+	-
A2-71	+	-	+	-	+	+	-	-	+	+	-	-	-	-
A2-95	-	-	-	-	+	+	-	-	+	+	+	-	-	-
A2-438	-	-	-	-	+	+	-	-	-	-	-	-	-	-
A2-441	-	-	-	-	+	-	-	-	+	+	-	-	-	-
A2-442	-	-	-	-	+	+	-	-	+	+	-	-	-	-

(+) growth; (-) no growth

3.4 Phylogenetic characterization of a major population of Rhodanobacter strains.

The phylogenetic analysis of the complete 16S rRNA gene sequences of the 6 strains of *Rhodanobacter* showed that they form 3 distinct groups (Fig. 2). One group is formed by strains A2-56, A2-61 and A2-60 and are closely related with *R. thiooxidans* LCS2^{T}. A second group of isolates grouped with the type strains of *R. umsongensis*

GR24-2^T, *R. ginsengisoli* GR17-7^T and *R. panaciterrae* LnR5-47^T. This group of strains showed a higher sequence similarity with *R. umsongensis* GR24-2^T (98.8 %). Strain A2-441 grouped with *R. terrae* GP18-1^T.

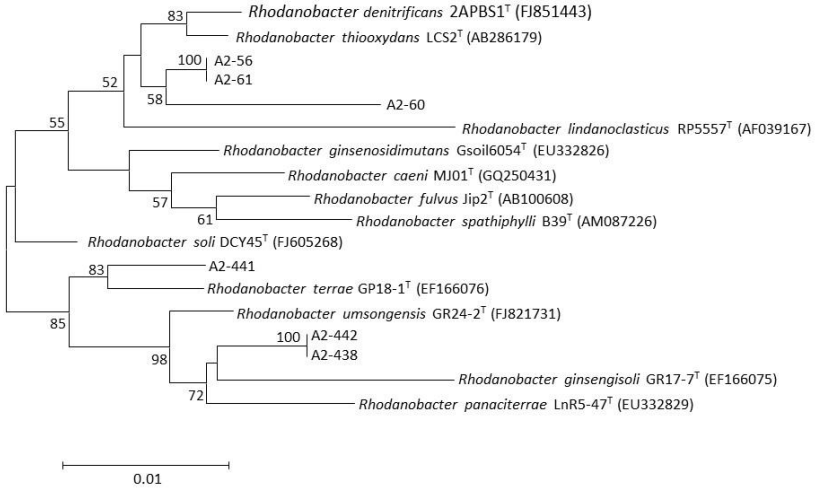

Fig. 2. Phylogenetic dendogram based on a comparison of the 16S rRNA gene sequences of *Rhodanobacter* strains isolated from A2- Poço das Cobras, and the closest phylogenetic relatives. The tree was created using the neighbour-joining method. The numbers on the tree indicate the percentages of bootstrap sampling, derived from 1000 replications. Scale bar, 1 inferred nucleotide substitution per 100 nucleotides.

4. Discussion

In this study, U (VI) and As (III) cultivable resistant populations were recovered in all environmental samples, independently of the amount of uranium. However, in Poço das Cobras, the sampling site with highest concentration of U (VI) (8.2 μM), the largest number of U (VI) cultivable resistant bacteria ($2.8x10^3$ CFU ml^{-1}) was recovered, when compared with the other sampling sites, demonstrating a stable uranium resistance population adapted to this stressed environment.

The phylogenetic analysis along with the metal resistant profile suggests that strains belonging to different species have different resistance profiles. The *R. thiooxidans* group was able to resist to U (VI) and Sb (V), which seems to be species-specific. Nevertheless, the resistance to Zn (II) and Cu (II) was only present in strains A2-60 and A2-61 of the same group. The group of *R. umsongensis* include strains resistant to U (VI), and strain A2-442 was also resistant to Sb (V). The strain that grouped with species *R. terrae* was the one that showed the lowest resistance to U(VI). However, there is no report that the type strains of the three species mentioned above have any particular resistance to heavy metals. The type strains of *R. umsongensis* and *R. terrae* were isolated from Korean ginseng field[12,13] and the type strain of *R. thiooxidans* was isolated from a biofilm on sulfur particles used in an autotrophic denitrification process[14].

Strains belonging to the *Rhodanobacter* genus have been isolated from different U contaminated environments[7,15]. What seems to be the driven factor for their success in these environments is not yet clarified. The strains from ORIFRC-Oak Ridge Integrated Field Research Challenge are characteristically resistant to low pH and are able to denitrify[15] which probably limit the *in situ* reoxidation of U(IV) by lowering the nitrate concentration[16]. Nevertheless, only Sousa and co-workers[7] demonstrated the direct ability of strain A2-61 from this genus to resist and to reduce U(VI) aerobically.

The recovery of this group of bacteria as one of the major group of isolates in our sampling site with a higher content of uranium corroborates the idea that strains from *Rhodanobacter* could have a major role in the fate of U in these environments.

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